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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/00, 33/53, 33/543, 33/566 | A1

(11) International Publication Number: WO 97/33169

(43) International Publication Date: 12 September 1997 (12.09.97)

(21) International Application Number: PCT/US97/03625

(22) International Filing Date: 7 March 1997 (07.03.97)

(30) Priority Data:
60/013,136 8 March 1996 (08.03.96) US

(71) Applicant *(for all designated States except US)*: SMITHKLINE BEECHAM CORPORATION [US/US]; One Franklin Plaza, Philadelphia, PA 19103 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DUNNINGTON, Damien, J. [US/US]; 23 Forsythia Court, Lafayette Hill, PA 19444 (US). YAMASHITA, Dennis, Shinji [US/US]; 703 Edgewood Road, King of Prussia, PA 19406 (US).

(74) Agents: DUSTMAN, Wayne, J. et al.; SmithKline Beecham Corporation, Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: A METHOD FOR DETERMINING THE AFFINITY OF PROTEINS FOR CHEMICAL AGENTS DURING SCREENING OF COMBINATORIAL LIBRARIES

(57) Abstract

Invented is a method for determining the affinity of protein targets for chemical agents during screening of combinatorial libraries. The libraries are synthesized on solid supports with a gradient of coupling sites, such that the target is presented with a range of compound concentrations. The distribution of protein bound to the immobilized compounds is measured by quantitative image analysis. Compounds are ranked in order of binding affinity by comparing their ability to bind across the gradient.

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A METHOD FOR DETERMINING THE AFFINITY OF PROTEINS FOR CHEMICAL AGENTS DURING SCREENING OF COMBINATORIAL LIBRARIES.

BACKGROUND OF THE INVENTION

In view of the large number of positives that are potentially observable during screening of large combinatorial libraries with target proteins, it is desirable to apply stringent criteria to select meaningful actives for follow-up. Such criteria may include one or more of the following: selectivity for one target versus a mutant form or closely related protein, binding affinity for the protein target, or binding to the active site of an enzyme versus allosteric or non-specific sites. Current methods for evaluating compounds against these criteria require production of the compound in a soluble form and separate testing. For example to measure the binding affinity of a agonist or antagonist ligand for a receptor it is necessary to incubate multiple aliquots of the compound with the receptor over a range of compound concentration. This is both tedious and time consuming and requires the availability of relatively large amounts of compound. There is need in the art for a rapid method of directly determining the binding affinity of compounds during the screening process. A novel method for determining the affinity of a compound for a protein target during screens of combinatorial libraries on solid phase supports is described below.

SUMMARY OF THE INVENTION

This invention relates to an improved method of biological evaluation of combinatorial libraries using a method that permits direct measurement of bonding affinity of compounds, to a biological target.

A prefered aspect of the invention provides a method for determining the affinity of target proteins for chemical agents during screening of combinatorial libraries which comprises: presenting a target protein with a gradient of compound concentrations on a solid support; and measuring the amount of bound target at each concentration. This invention also relates to compounds identified using the this method. Preferably the amount of bound target is measured by imaging the distribution of protein bound to the support.

This invention also relates to a support for solid-phase compound synthesis bearing a non-uniform distribution of chemical coupling sites and to compound libraries synthesized on this support.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts a fabrication of polymer disks with a radial distribution of coupling densities by coaxial extrusion. As used in Fig. 1, Sc is the equivalent local concentration of coupling sites.

Fig. 2 depicts a fabrication of rods carrying a linear gradient of coupling densities.

DETAILED DESCRIPTION

The principle of this method is to present the protein target with a range of spatially localized compound concentrations and measure the amount of bound target at each concentration. In the case of solid phase libraries, this is achieved by varying the coupling density of the compound to the support to create a gradient of surface compound density. Binding of protein to the compound gradients is measured by using reagents that generate an optical readout corresponding to the bound protein and subsequent imaging the distribution of bound protein with compound density. The key components of this invention are a solid support fabricated with a gradient of chemical coupling sites for compound attachment, a protein target of pharmaceutical interest, accessory reagents for generation of an optical signal and a quantitative imaging apparatus for measurement of the optical signal.

The solid phase support is critical for this method and is fabricated with a predictable gradient of chemical coupling sites. Gradients are constructed on disks, rods, ellipsoids, beads or other substrate geometries. The preferred configurations are disks or rods (Figs. 1, 2). The number of chemical coupling sites on a given polymer is controlled by 'doping' of chemically selective linkers, photochemical activation/inactivation of sensitized coupling groups or controlled blocking of unwanted sites by chemical means, such as removing a reactive site from a Wang linker with an alkylating agent such as methyl iodide or removing a reactive site from a Merrifield linker with an alkoxide such as potassium methoxide or removing a reactive amine-containing linker with an acylating reagent such as benzoyl chloride. Preferred substrates are Rapp Tentagel, a polyethylene glycol polymer, or Perseptive polystyrene-polyethylene glycol polymer because they are wettable with aqueous reagents.

Disks carrying discontinuous gradients of coupling densities are fabricated by co-axial extrusion of cylinders of polymer carrying defined densities of coupling sites (Fig. 1.), followed by sectioning. Each layer of polymer is doped with a known

concentration of linker prior to extrusion by addition of a specific concentration of linker to the polymer. For example, if one wanted a polyethylene glycol (PEG) based resin with 3.3 mM concentration of compound present, one would take bromo ethyl Rapp Tentagel and react it with a 10:1 ratio of para- hydroxy toluene and para-hydroxy benzyl alcohol with a base such as sodium hydride in a solvent such as DMF resulting in a 10:1 dilution of available Wang-like linker sites. If one wanted a polyethylene glycol based resin with 0.33 mM concentration of compound present, one would take bromo ethyl Rapp Tentagel and react it with a 10:1 ratio of para-hydroxy toluene and para-hydroxy benzyl alcohol with a base such as sodium hydride in a solvent such as DMF resulting in a 100:1 dilution of available Wang-like linker sites. If one wanted a polyethylene glycol based resin with 33 microM concentration of compound present, one would take bromo ethyl Rapp Tentagel and react it with a 1000:1 ratio of para-hydroxy toluene and para-hydroxy benzyl alcohol with a base such as sodium hydride in a solvent such as DMF resulting in a 1000:1 dilution of available Wang-like linker sites.

Alternatively, one could dilute the number of available linker sites in the construction of a Perseptive PEG-polystyrene-like particle in an analogous way by using a 10:1 ratio of unfunctionalized PEG to functionalized PEG, during the attachment step of the PEG units to the polystyrene particles. For a 10 fold further dilution in linker sites, use a 100:1 ratio of unfunctionalized PEG to functionalized PEG, during the attachment step of the PEG units to the polystyrene particles. For a 100 fold further dilution in linker sites, use a 1000:1 ratio of unfunctionalized PEG to functionalized PEG, during the attachment step of the PEG units to the polystyrene particles. Alternatively, rods of polymer carrying photocleavable linkers can be extruded and during extrusion, the light level can be varied to inactivate the linkers in the desired spatial configuration (Fig. 2). This approach allows fabrication of linear or exponential gradients. Another method is to use photolithographic techniques to photoactivate or photoinactivate light-sensitive linkers in the desired gradient pattern on a photoreactive substrate, followed by die stamping to produce particles carrying the imprinted pattern. The gradient may be linear, non-linear or discontinuous and the density range is chosen according to the needs of the screen. For example, if compounds with affinities for the protein target in the 10-100 nM range are desired, the gradient is fabricated to achieve an equivalent local concentration of compound ranging from 1 to 1000 nM. The area occupied by the gradient is dependent on the limit of resolution of the imaging device. Typically, this limit is approximately 100 um for macro imaging with most CCD cameras, in which case, the gradient must occupy an area of at least 0.1 x 1 mm for a rectangular

gradient, to allow resolution of 100 nM steps in equivalent compound concentration. The use of high density CCD arrays and/or magnifying optics will permit higher resolution or smaller gradient areas at the expense of throughput, the latter being limited by the field area that can be imaged. The equivalent local concentration of compound is estimated from the concentration of coupling sites within the polymer. For example, unmodified Rapp Tentagel contains ~300 pmol coupling sites per 260 microM (swollen) bead. This corresponds to a compound concentration within the polymer of ~33 mM, assuming a monovalent linker is used and all the coupling sites are occupied. The nature of the linker is chosen according to the desired chemistry of the subsequent combinatorial synthesis. Examples of well known linkers are those of Wang or Merrifield.

Once a collection of such particles has been fabricated, synthesis of the library is done by conventional combine and split protocols. The result is a collection of particles, each with a gradient of local density of an individual compound. The particles are screened against a protein target by addition of the particles to a solution of the protein target and by measurement of the optical signal associated with the protein. The protein target may be soluble or membrane bound. It may be directly labelled with a substance capable of generating an optical signal. Preferably, the optical signal is fluorescence or luminescence. Fluorophores are attached to proteins by chemical means. A well known example is the use of fluorescein isothipcyanate which attaches fluorescein to amino groups in the protein. Alternatively, the protein may be indirectly labelled by providing a fluorescently labelled antibody that recognises the protein itself, or a suitable tag incorporated within the sequence of the protein. These and related techniques for labelling proteins are well known to those skilled in the art. For membrane-bound targets, lipophilic fluorescent dyes are available from commercial sources, for example, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine, available from Molecular Probes of Eugene, Or. Such dyes dissolve in membrane lipids and are used to label vesicles carrying receptors or other membrane-associated protein targets.

Prior to the addition of the protein target, the particles may be treated with a high concentration of a blocking reagent to occupy non-specific protein binding sites. Examples of such blocking reagents are 1% bovine serum albumin or casein. After addition of the protein target, the particle suspension is incubated to attain binding equilibrium. The incubation conditions will vary for each protein target, but in general, 2 hours at 37 degrees Celsius will suffice for most targets. The optimum protein concentration will depend on the sensitivity of the optical detection device

and the binding affinity and number of active compounds in the library and may be determined empirically.

Following incubation, the particles are washed with a suitable buffer, for example, 10 mM HEPES pH 7.6, 0.15 mM sodium chloride, 0.1% NP-40. The particles are then spread onto a filter paper and imaged. The optical signal may be detected by using a CCD camera, such as the Tundra instrument from Imaging Research Inc., St Catherine's, Ontario, Canada. Positive particles are selected by measuring the decrease of optical signal along the axis of the gradient. Compounds that bind the protein with high affinity will generate a signal at low compound densities, therefore the signal will extend into the regions of the gradient with low densities of such compounds. By comparison of the optical signal of each particle at a given point within the gradient, compounds are ranked in order of binding affinity.

After selection of particles carrying high affinity ligands is done as above, the compounds are identified. This may be done by cleaving the compound from each particle and subjecting it to analysis by mass spectrometry. Alternatively, the compounds may be tagged with specific chemical markers during synthesis, such that the nature of the compound is encoded by the tag. Methods for such tagging are well known to those skilled in the art. An example of mass spectrometric compound identification is : A mass spectrometric solution to the address problem of combinatorial libraries, Brummel-CL; Lee-IN; Zhou-Y; Benkovic-SJ; Winograd-N, Science. 1994 264 (5157): 399-402.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. While the preferred embodiments of the invention are illustrated by the above, it is to be understood that the invention is not limited to the precise instructions herein disclosed and the right to all modifications coming within the scope of the following claims is reserved.

What is claimed is:

1. A method for determining the affinity of target proteins for chemical agents during screening of combinatorial libraries which comprises:

a) presenting a target protein with a gradient of compound concentrations on a solid support; and

b) measuring the amount of bound target at each concentration

2. The method of claim 1 in which the amount of bound target is measured by imaging the distribution of protein bound to the support.

3. A support for solid-phase compound synthesis bearing a non-uniform distribution of chemical coupling sites.

4. compound libraries synthesized in the support of claim 3.

5. Compounds identified using the methods of claim 1.

Fig. 1

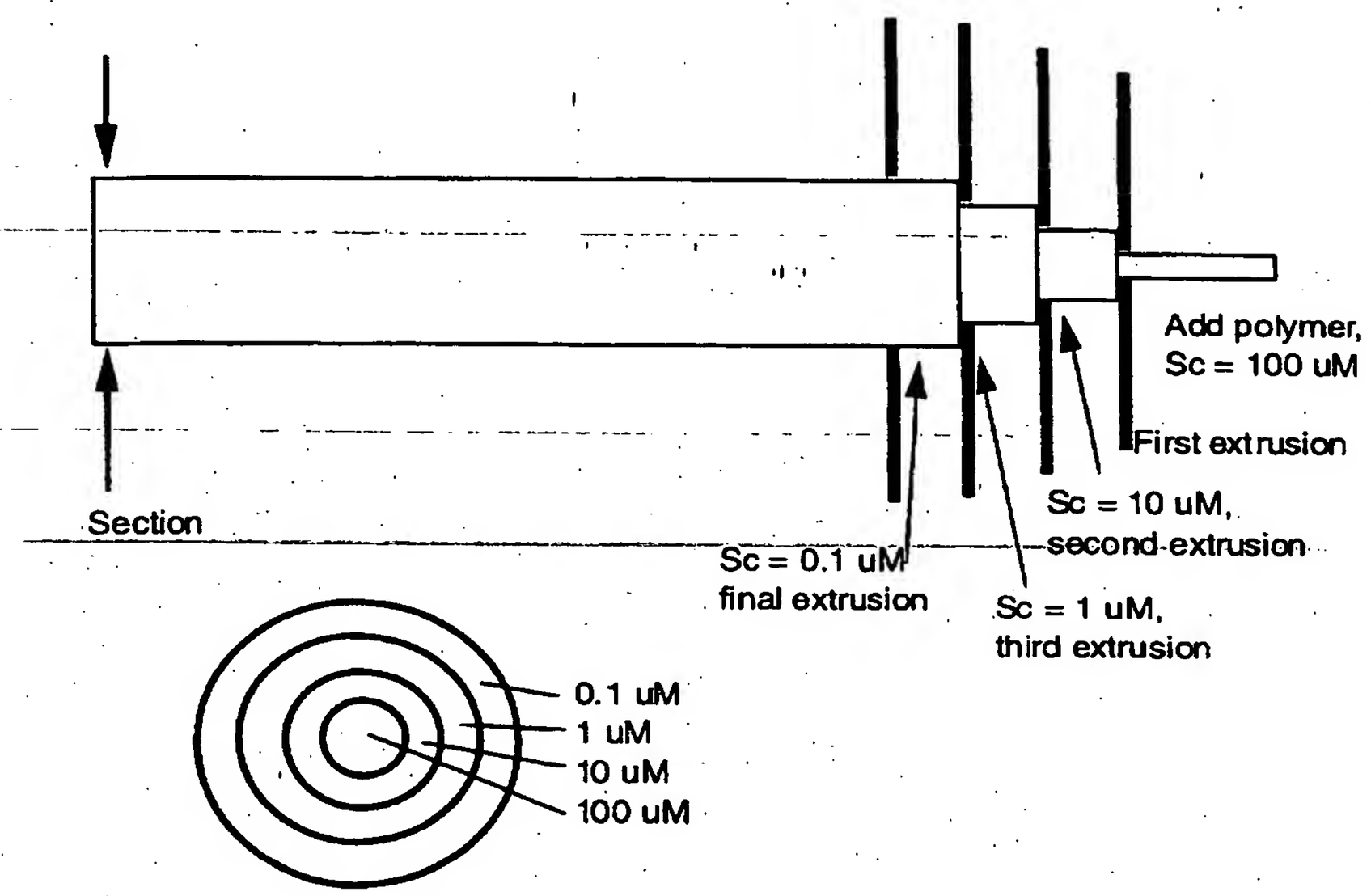
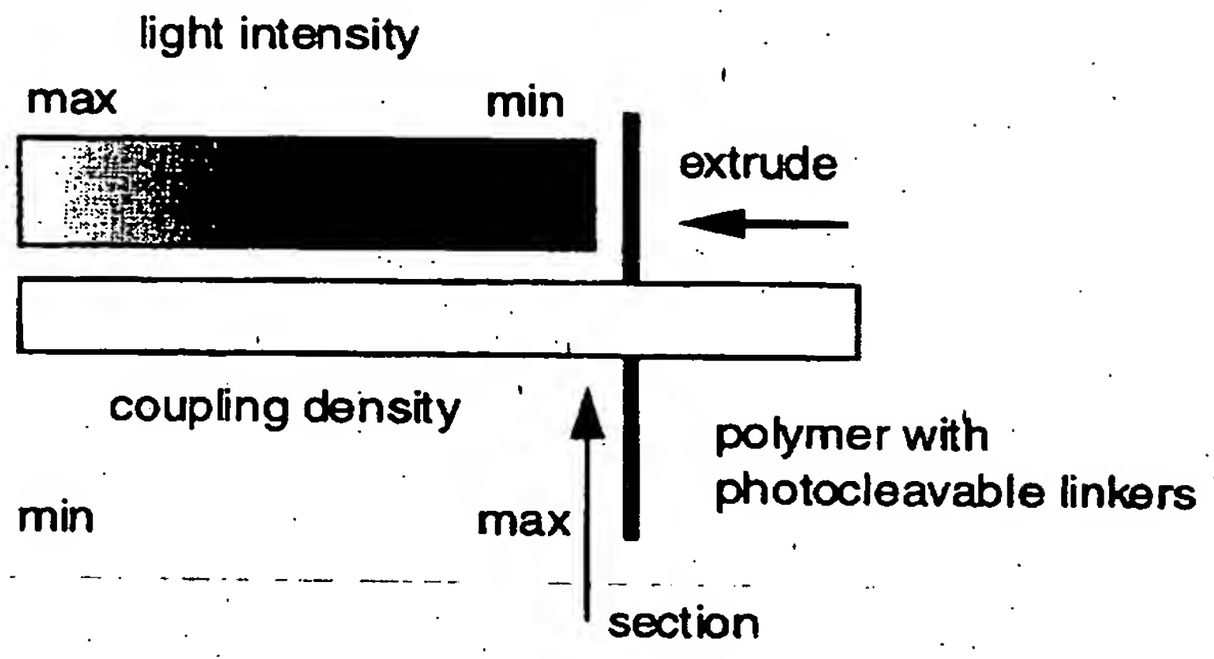

Add polymer,
Sc = 100 uM
First extrusion
Sc = 10 uM,
second extrusion
Sc = 1 uM,
third extrusion
Sc = 0.1 uM
final extrusion
Section
0.1 uM
1 uM
10 uM
100 uM

FIG.2


light intensity
max
min
extrude
coupling density
min
max
polymer with
photocleavable linkers
section

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03625

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/00, 33/53, 33/543, 33/566
US CL :436/86, 501, 518; 435/7.1, 7.8; 530/333

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/86, 501, 518, 523, 524, 528; 435/7.1, 7.8, 7.92; 530/333, 334

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN/CA, BIOSIS, MEDLINE, WPIDS, EUROPATFULL
search terms: combinatorial library, target protein, gradient, concentration, solid phase, support

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US 5,545,568 (ELLMAN) 13 August 1996, column 2, lines 21-24 and column 70, lines 29-33.	3-4
A, P		1-2, 5
Y, P	EP 0742438 (J.A. BUETTNER) 13 November 1996, column 17, lines 16-28.	1-5
Y, P	US 5,591,646 (HUDSON et al.) 07 January 1997, column 130, lines 39-59 and column 132, lines 1-11.	1-5
A	US 5,143,854 (PIRRUNG et al.) 01 September 1992, column 3, lines 39-54 and column 4, lines 9-14.	1-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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CORRECTED VERSION*

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(57) Abstract

Invented is a method for determining the affinity of protein targets for chemical agents during screening of combinatorial libraries. The libraries are synthesized on solid supports with a gradient of coupling sites, such that the target is presented with a range of compound concentrations. The distribution of protein bound to the immobilized compounds is measured by quantitative image analysis. Compounds are ranked in order of binding affinity by comparing their ability to bind across the gradient.

* (Referred to in PCT Gazette No. 49/1997, Section II)

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A METHOD FOR DETERMINING THE AFFINITY OF PROTEINS FOR CHEMICAL AGENTS DURING SCREENING OF COMBINATORIAL LIBRARIES.

BACKGROUND OF THE INVENTION

In view of the large number of positives that are potentially observable during screening of large combinatorial libraries with target proteins, it is desirable to apply stringent criteria to select meaningful actives for follow-up. Such criteria may include one or more of the following: selectivity for one target versus a mutant form or closely related protein, binding affinity for the protein target, or binding to the active site of an enzyme versus allosteric or non-specific sites. Current methods for evaluating compounds against these criteria require production of the compound in a soluble form and separate testing. For example to measure the binding affinity of a agonist or antagonist ligand for a receptor it is necessary to incubate multiple aliquots of the compound with the receptor over a range of compound concentration. This is both tedious and time consuming and requires the availability of relatively large amounts of compound. There is need in the art for a rapid method of directly determining the binding affinity of compounds during the screening process. A novel method for determining the affinity of a compound for a protein target during screens of combinatorial libraries on solid phase supports is described below.

SUMMARY OF THE INVENTION

This invention relates to an improved method of biological evaluation of combinatorial libraries using a method that permits direct measurement of bonding affinity of compounds, to a biological target.

A prefered aspect of the invention provides a method for determining the affinity of target proteins for chemical agents during screening of combinatorial libraries which comprises: presenting a target protein with a gradient of compound concentrations on a solid support; and measuring the amount of bound target at each concentration. This invention also relates to compounds identified using the this method. Preferably the amount of bound target is measured by imaging the distribution of protein bound to the support.

This invention also relates to a support for solid-phase compound synthesis bearing a non-uniform distribution of chemical coupling sites and to compound libraries synthesized on this support.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a depicts a fabrication of polymer disks with a radial distribution of coupling densities by coaxial extrusion. As used in Fig. 1a, Sc is the equivalent local concentration of coupling sites.

Fig. 1b is a frontal view of the coaxial extrusion of Fig. 1a.

Fig. 2 depicts a fabrication of rods carrying a linear gradient of coupling densities.

DETAILED DESCRIPTION

The principle of this method is to present the protein target with a range of spatially localized compound concentrations and measure the amount of bound target at each concentration. In the case of solid phase libraries, this is achieved by varying the coupling density of the compound to the support to create a gradient of surface compound density. Binding of protein to the compound gradients is measured by using reagents that generate an optical readout corresponding to the bound protein and subsequent imaging the distribution of bound protein with compound density. The key components of this invention are a solid support fabricated with a gradient of chemical coupling sites for compound attachment, a protein target of pharmaceutical interest, accessory reagents for generation of an optical signal and a quantitative imaging apparatus for measurement of the optical signal.

The solid phase support is critical for this method and is fabricated with a predictable gradient of chemical coupling sites. Gradients are constructed on disks, rods, ellipsoids, beads or other substrate geometries. The preferred configurations are disks or rods (Figs. 1, 2). The number of chemical coupling sites on a given polymer is controlled by 'doping' of chemically selective linkers, photochemical activation/inactivation of sensitized coupling groups or controlled blocking of unwanted sites by chemical means, such as removing a reactive site from a Wang linker with an alkylating agent such as methyl iodide or removing a reactive site from a Merrifield linker with an alkoxide such as potassium methoxide or removing a reactive amine-containing linker with an acylating reagent such as benzoyl chloride. Preferred substrates are Rapp Tentagel, a polyethylene glycol polymer, or Perseptive polystyrene-polyethylene glycol polymer because they are wettable with aqueous reagents.

Disks carrying discontinuous gradients of coupling densities are fabricated by co-axial extrusion of cylinders of polymer carrying defined densities of coupling sites (Fig. 1.), followed by sectioning. Each layer of polymer is doped with a known

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Alternatively, one could dilute the number of available linker sites in the construction of a Perseptive PEG polystyrene like particle in an analogous way by using a 10:1 ratio of unfunctionalized PEG to functionalized PEG, during the attachment step of the PEG units to the polystyrene particles. For a 10 fold further dilution in linker sites, use a 100:1 ratio of unfunctionalized PEG to functionalized PEG, during the attachment step of the PEG units to the polystyrene particles. For a 100 fold further dilution in linker sites, use a 1000:1 ratio of unfunctionalized PEG to functionalized PEG, during the attachment step of the PEG units to the polystyrene particles. Alternatively, rods of polymer carrying photocleavable linkers can be extruded and during extrusion, the light level can be varied to inactivate the linkers in the desired spatial configuration (Fig. 2). This approach allows fabrication of linear or exponential gradients. Another method is to use photolithographic techniques to photoactivate or photoinactivate light-sensitive linkers in the desired gradient pattern on a photoreactive substrate, followed by die stamping to produce particles carrying the imprinted pattern. The gradient may be linear, non-linear or discontinuous and the density range is chosen according to the needs of the screen. For example, if compounds with affinities for the protein target in the 10-100 nM range are desired, the gradient is fabricated to achieve an equivalent local concentration of compound ranging from 1 to 1000 nM. The area occupied by the gradient is dependent on the limit of resolution of the imaging device. Typically, this limit is approximately 100 um for macro imaging with most CCD cameras, in which case, the gradient must occupy an area of at least 0.1 x 1 mm for a rectangular

gradient, to allow resolution of 100 nM steps in equivalent compound concentration. The use of high density CCD arrays and/or magnifying optics will permit higher resolution or smaller gradient areas at the expense of throughput, the latter being limited by the field area that can be imaged. The equivalent local concentration of compound is estimated from the concentration of coupling sites within the polymer. For example, unmodified Rapp Tentagel contains ~300 pmol coupling sites per 260 microM (swollen) bead. This corresponds to a compound concentration within the polymer of ~33 mM, assuming a monovalent linker is used and all the coupling sites are occupied. The nature of the linker is chosen according to the desired chemistry of the subsequent combinatorial synthesis. Examples of well known linkers are those of Wang or Merrifield.

Once a collection of such particles has been fabricated, synthesis of the library is done by conventional combine and split protocols. The result is a collection of particles, each with a gradient of local density of an individual compound. The particles are screened against a protein target by addition of the particles to a solution of the protein target and by measurement of the optical signal associated with the protein. The protein target may be soluble or membrane bound. It may be directly labelled with a substance capable of generating an optical signal. Preferably, the optical signal is fluorescence or luminescence. Fluorophores are attached to proteins by chemical means. A well known example is the use of fluorescein isothiocyanate which attaches fluorescein to amino groups in the protein. Alternatively, the protein may be indirectly labelled by providing a fluorescently labelled antibody that recognises the protein itself, or a suitable tag incorporated within the sequence of the protein. These and related techniques for labelling proteins are well known to those skilled in the art. For membrane-bound targets, lipophilic fluorescent dyes are available from commercial sources, for example, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine, available from Molecular Probes of Eugene, Or. Such dyes dissolve in membrane lipids and are used to label vesicles carrying receptors or other membrane-associated protein targets.

Prior to the addition of the protein target, the particles may be treated with a high concentration of a blocking reagent to occupy non-specific protein binding sites. Examples of such blocking reagents are 1% bovine serum albumin or casein. After addition of the protein target, the particle suspension is incubated to attain binding equilibrium. The incubation conditions will vary for each protein target, but in general, 2 hours at 37 degrees Celsius will suffice for most targets. The optimum protein concentration will depend on the sensitivity of the optical detection device

and the binding affinity and number of active compounds in the library and may be determined empirically.

Following incubation, the particles are washed with a suitable buffer, for example, 10 mM HEPES pH 7.6, 0.15 mM sodium chloride, 0.1% NP-40. The particles are then spread onto a filter paper and imaged. The optical signal may be detected by using a CCD camera, such as the Tundra instrument from Imaging Research Inc., St Catherine's, Ontario, Canada. Positive particles are selected by measuring the decrease of optical signal along the axis of the gradient. Compounds that bind the protein with high affinity will generate a signal at low compound densities, therefore the signal will extend into the regions of the gradient with low densities of such compounds. By comparison of the optical signal of each particle at a given point within the gradient, compounds are ranked in order of binding affinity.

After selection of particles carrying high affinity ligands is done as above, the compounds are identified. This may be done by cleaving the compound from each particle and subjecting it to analysis by mass spectrometry. Alternatively, the compounds may be tagged with specific chemical markers during synthesis, such that the nature of the compound is encoded by the tag. Methods for such tagging are well known to those skilled in the art. An example of mass spectrometric compound identification is : A mass spectrometric solution to the address problem of combinatorial libraries, Brummel-CL; Lee-IN; Zhou-Y; Benkovic-SJ; Winograd-N, Science. 1994 264 (5157): 399-402.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. While the preferred embodiments of the invention are illustrated by the above, it is to be understood that the invention is not limited to the precise instructions herein disclosed and the right to all modifications coming within the scope of the following claims is reserved.

What is claimed is:

1. A method for determining the affinity of target proteins for chemical agents during screening of combinatorial libraries which comprises:

a) presenting a target protein with a gradient of compound concentrations on a solid support; and

b) measuring the amount of bound target at each concentration

2. The method of claim 1 in which the amount of bound target is measured by imaging the distribution of protein bound to the support.

3. A support for solid-phase compound synthesis bearing a non-uniform distribution of chemical coupling sites.

4. compound libraries synthesized in the support of claim 3.

5. Compounds identified using the methods of claim 1.

Figure 1a

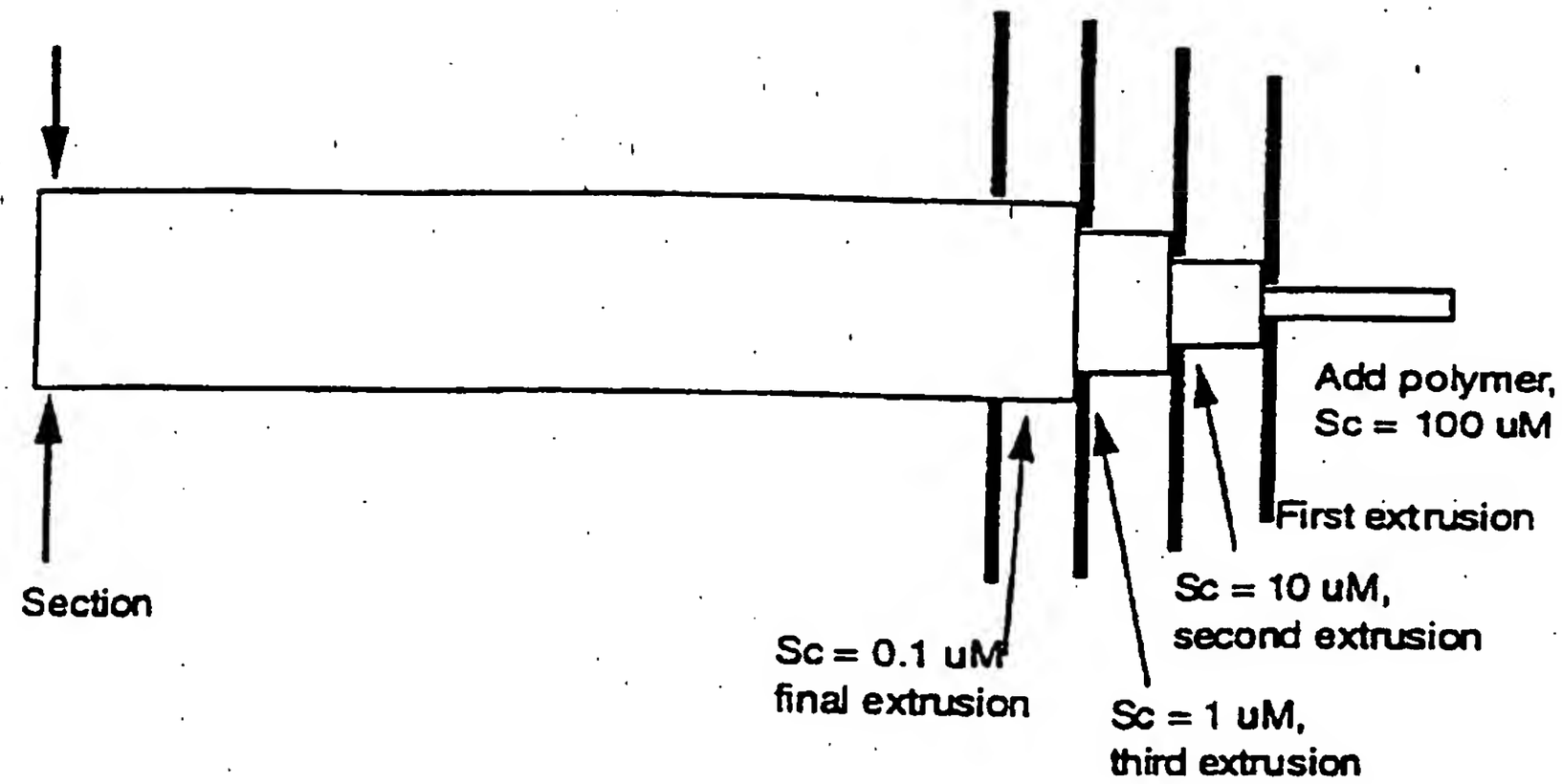


Figure 1b

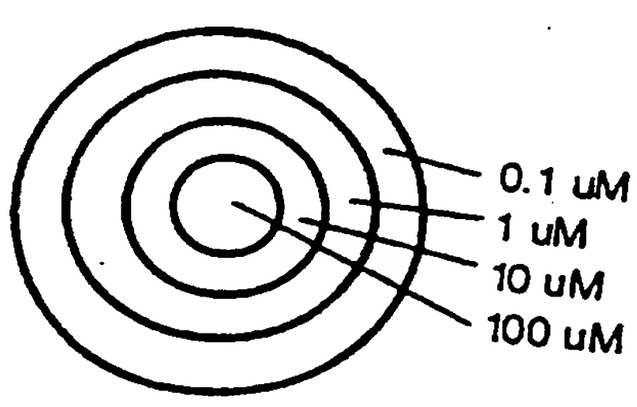
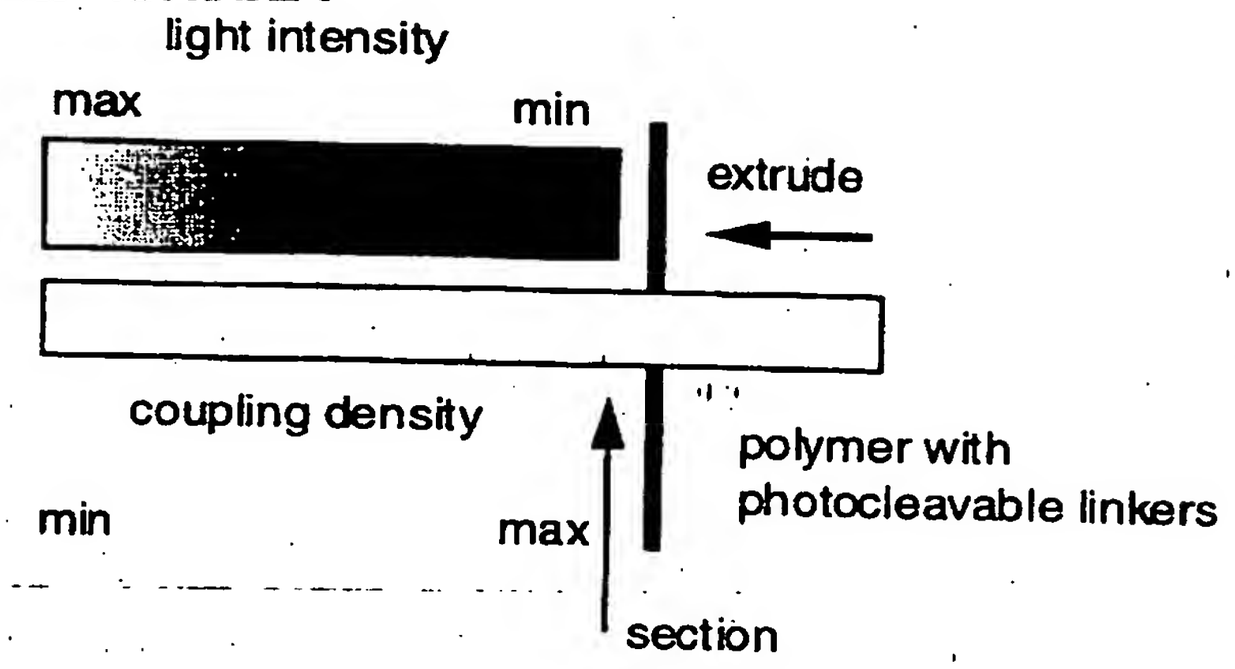


FIG.2


light intensity
max
min
extrude
coupling density
min
max
polymer with
photocleavable linkers
section

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03625

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/00, 33/53, 33/543, 33/566
US CL :436/86, 501, 518; 435/7.1, 7.8; 530/333

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/86, 501, 518, 523, 524, 528; 435/7.1, 7.8, 7.92; 530/333, 334

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN/CA, BIOSIS, MEDLINE, WPIDS, EUROPATFULL
search terms: combinatorial library, target protein, gradient, concentration, solid phase, support

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P --- A, P	US 5,545,568 (ELLMAN) 13 August 1996, column 2, lines 21-24 and column 70, lines 29-33.	3-4 --- 1-2, 5
Y, P	EP 0742438 (J.A. BUETTNER) 13 November 1996, column 17, lines 16-28.	1-5
Y, P	US 5,591,646 (HUDSON et al.) 07 January 1997, column 130, lines 39-59 and column 132, lines 1-11.	1-5
A	US 5,143,854 (PIRRUNG et al.) 01 September 1992, column 3, lines 39-54 and column 4, lines 9-14.	1-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 APRIL 1997	14 MAY 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MAUREEN WALLENHORST Telephone No. (703) 308-0651

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